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14. ABSTRACT
Prostate cancer is a difficult disease to treat due to its molecular heterogeneity and diverse clinical outcomes. Current therapies for treating and diagnosing prostate cancer are unsatisfactory, suggesting that new strategies and molecular markers are greatly needed. Tumor cells express specific cell surface receptor complexes for rapid growth and survival. Specific receptor-ligand complexes have profound biological functions such as cell signaling and growth. For example, androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy. We propose to identify new receptor-ligand pairs for prostate cancer. We have developed a sophisticated targeting system to probe the tumor vasculature in vivo by phage display technology. We plan to inject phage peptides libraries into prostate tumor-bearing mice to identify specific peptides targeting to the tumor and not to the normal tissues. The tumor-specific peptides will be recovered and analyzed by molecular and biochemical methods. The tumor-specific peptides will be used as a bait to identify and clone the binding receptors by affinity chromatography and biochemical cell fractionation approaches. If we are successful, we will identify new biologically relevant receptor-ligand pairs that may be developed for therapeutic applications for prostate cancer.

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INTRODUCTION

Prostate cancer is a difficult disease to treat due to its molecular heterogeneity and diverse clinical outcomes. Current therapies for treating and diagnosing prostate cancer are unsatisfactory, suggesting that new strategies and molecular markers are greatly needed. Tumor cells express specific cell surface receptor complexes for rapid growth and survival. Specific receptor-ligand complexes have profound biological functions such as cell signaling and growth. For example, androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy. It is important that more such complexes are identified for this disease. We propose to identify specific receptor-ligand pairs for prostate cancer. We have developed a sophisticated targeting system to probe the tumor vasculature in vivo by phage display technology. We plan to inject phage peptides libraries into prostate tumor-bearing mice to identify specific peptides targeting to the tumor and not to the normal tissues. The tumor-specific peptides will be recovered and analyzed by molecular and biochemical methods. The tumor-specific peptides will be used as a bait to identify and clone the binding receptors by affinity chromatography and biochemical cell fractionation approaches. If we are successful, we will identify new biologically relevant receptor-ligand pairs that may be developed into diagnostic and/or therapeutic applications for prostate cancer.

BODY

Background:

Prostate cancer is the second leading cause of cancer dearth in men and it is estimated that one in six men will develop this disease during their lifetime (1). The cause of the disease is largely unknown. This is also compounded by the fact the disease is heterogeneous with diverse clinical outcomes. Studies have shown that tumors are heterogeneous comprising sub-population of tumor cells with different molecular properties and genetic alterations (2-4). Some of these different properties include growth rate, metastasis, resistance to cytotoxic drugs, and cell surface receptors (3). Moreover, the tumor microenvironment is extremely complex consisting of many cell types that can crosstalk with each other by activating and inactivating cell surface receptors (5). Tumor cells express specific cell surface receptors that can interact with growth factors and cytokines for rapid growth, survival, and cell signaling to the extracellular environment. Specific receptor-ligand complexes can have profound biological functions. For example, in the case for prostate cancer, it is well known that androgen-androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy (6, 7). The complex specifically activates transcription of androgen-regulated genes and promotes cellular proliferation, survival, and differentiation (8, 9). However, there are a limited number of receptor-ligand pairs that have been thus far identified for prostate cancer. We propose to identify specific receptor-ligand pairs for prostate cancer by in vivo phage display. This approach has not been explored for targeting prostate tumor in vivo. Identifying the molecular receptor-ligand complexes during tumor development is an important step towards developing new diagnostic markers and molecular therapeutic targets for prostate cancer.

Statement of work:

Task 1. To isolate and characterize peptides targeting prostate tumor cells in vivo by phage peptide libraries (1-18 months).

Summary of Task 1:

- In vivo screening in tumor-bearing mice (human prostate cancer xenografts) by phage peptide libraries have been successfully completed.
- Specific phage peptides have been identified and isolated from the tumor-bearing mice injected with the phage libraries.
- Few of the phage peptides have been characterized for their binding and inhibition properties on prostate cancer cell lines including DU145, PC-3, and LNCaP. Also, the phage peptides have been tested in the tumor-bearing mice.
- Localization and immunohistochemical analysis have been performed for some of the specific phage peptides on tissue samples from the tumor-bearing mice and prostate cancer cell lines.

We have successfully completed Task 1 as previously reported in the first annual report.

Task 2. To identify and validate molecular receptors binding to the tumor homing peptides (18-36 months).

Summary of Task 2:

- Specific tumor-homing phage peptides have been identified and analyzed by protein databases (NCBI) for biologically relevant receptor leads for prostate cancer.
- A specific peptide was selected based on biochemical and functional assays. Affinity chromatography and biochemical cell fraction was used to identify the corresponding receptor.
- The corresponding receptor has been identified as being CRKL. We are in the process of characterizing CRKL in the context of prostate cancer.
- We have shown that one of its binding partners is an integrin receptor called β_1 integrin. We have recently identified that CRKL also binds to and functionally interacts with the androgen receptor. In addition, we also show that AR-CRKL complex co-localizes in the prostate cancer cells by immunofluorescence analysis. We are still characterizing and validating these complexes in prostate cancer cells.

Specifically in this report we have performed the following experiments to functionally characterize and validate CRKL in prostate cancer.

We have shown that CRKL is important in prostate cancer and interacts with β_1 integrin on the cell surface, we next wanted to validate the downstream signalling mechanisms of CRKL activation and if this might have any direct functional consequences with the androgen receptor. To our surprise we found that in addition to the β_1 integrin, we found that CRKL and AR are in a complex (Fig. 1). This is an important find because it demonstrates for the first time that an intracellular signalling adaptor molecule such as CRKL could interact with AR. This is in contrast to the already known partners of the AR, which are nuclear proteins[15,16].

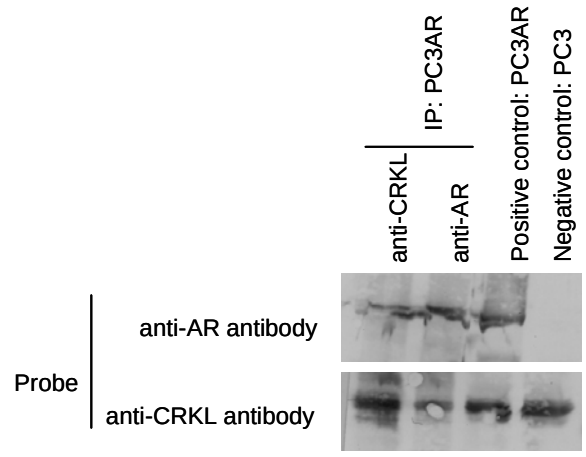

Figure 1. AR and CRKL complex. Co-immunoprecipitation from PC3AR cells.

To understand the functional role of the AR-CRKL complex, a CRKL expression construct was investigated in an AR transcriptional reporter assay. We first co-transfected CRKL and an AR reporter into COS cells and measured its activity. We found that CRKL could transcriptionally activate the AR when stimulated with the hormone mibolerone (Fig. 1a). This result prompted us to further examine this event in prostate cancer cells. A similar transactivation was observed in androgen-independent PC3 cells (Fig. 1b) and interestingly, also found that CRKL was phosphorylated (Fig. 1b, inset) suggesting that phosphorylated CRKL is recruited to the AR complex and acts as a co-activator.

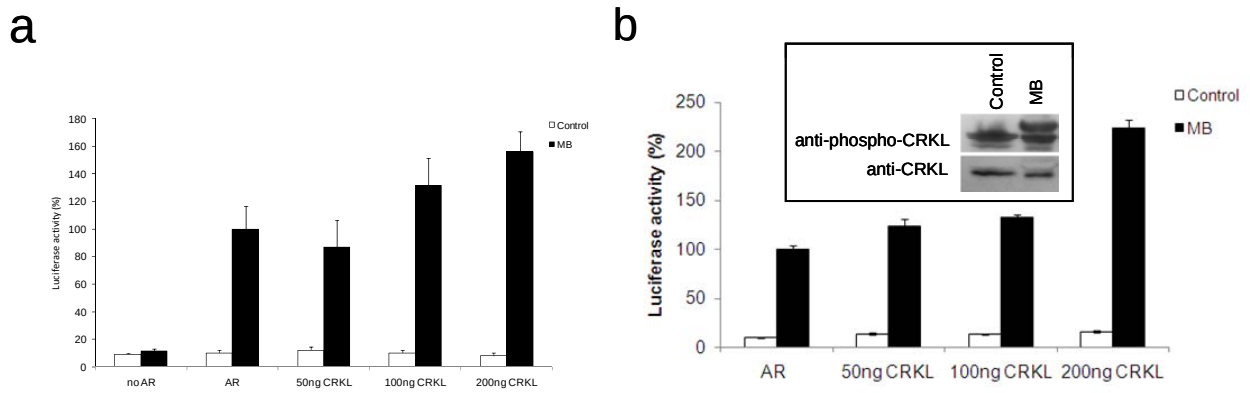

Figure 2. Functional reporter assay. AR and CRKl were co-transfected into PC3 prostate cancer cells and its transcriptional activity was measured. Cell lysates were used to probe for phosphorylated CRKL.

Encouraged by these initial results and since CRKL is distributed both in the cytoplasm and nucleus (10,11), we next wanted to determine the sub-cellular distribution of AR-CRKL complex in prostate cancer cells. A significant co-localization between CRKL and AR was observed in PC3 cells when co-transfected with the AR and CRKL constructs or in PC3AR cells when transfected with the CRKL construct (Fig. 3a,b). A similar staining pattern was also observed in the LNCaP cells (Fig. 3c). When stimulated with the hormone mibolerone, CRKL localised mostly to the nucleus, whereas in the presence of the AR inhibitor casodex, a more diffuse distribution was observed. It is possible that the AR and CRKL may form a complex in the cytoplasm when stimulated by the steroid and translocate into the cell nucleus. Since CRKL has a nuclear export signal (12) it has the capability of exiting back into the cytoplasm where it can interact with the AR. Taken together, these data provide evidence of co-localisation of AR and CRKL in androgen-independent prostate cancer cells.

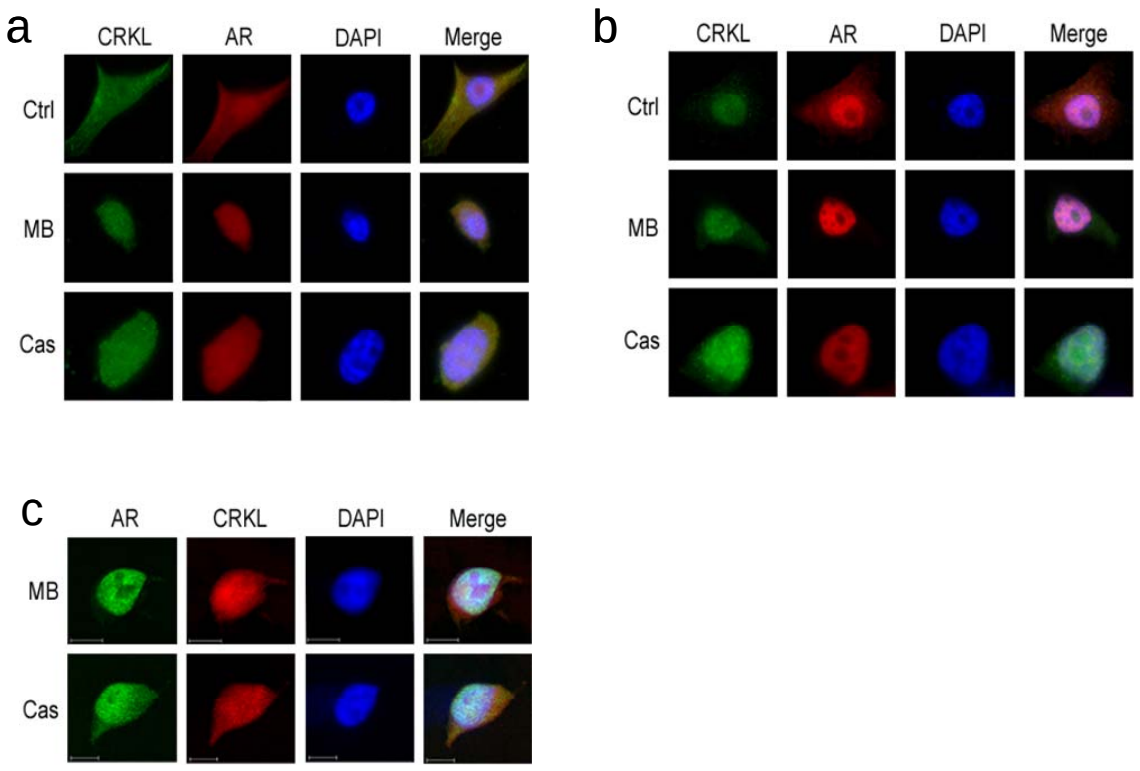

Figure 3. Co-localisation of AR and CRKL complex. PC3 cells were co-transfected with AR and CRKL (**a**), PC3AR cells transfected with CRKL (**b**), and LNCaP cells transfected with CRKL (**c**). The transfected cells were stimulated with milbolerone or inhibited by casodex.

In summary:

We have identified a couple of new receptor-ligand complexes in prostate cancer. The association between β_1 integrin/CRKL and AR/CRKL are novel discoveries and this is the first report of such finding. We plan to complete the functional characterization of this association in the context of the disease. The results from Task 1 and 2 has been submitted and accepted by a peer-review journal *PNAS* (please see accomplishment section). We are grateful for the continue funding from the Department of Defense (DOD). This work could not have been possible without the funding from DOD.

Materials and Methods:

Cell Culture

PC3 and LNCaP (American Type Culture Collection) were cultured in RPMI-1640 (Sigma) supplemented with 100 units/ml penicillin, 0.1mg/ml streptomycin, 2mmol/L glutamine (Sigma) and 10% fetal bovine serum (Labtech International). PC3wtAR cells (13) were grown in the presence of 4μg/ml of Geneticin (Gibco). Androgen-free culture conditions were carried out in phenol red free DMEM or RPMI supplemented with charcoal-stripped fetal bovine serum (Labtech International).

Reporter assay

The following plasmids have been described previously: pSG5-SRC-1e (14)**,** pSVAR (ΔLBD) (a.a 1-653) (15) and pCDNA-CRKL (16). The following were kind gifts: pSVAR from Brinkmann A (Rotterdam), TAT-GRE-EIB-Luc from Jenster G (Rotterdam). Cells were cultured in 24-well plates for 24 hours followed by transfection using FuGENE6 (Roche Diagnostics). The transfected DNA (measured in nanograms per well) included pSG5 or pCDNA3.1 control plasmids to standardise the amounts of DNA, the reporter TAT-GRE-E1B-Luc (500ng), pdmLacZ-β-Gal (250ng) and the vectors pSVAR (50ng), pSVAR- ΔLBD (50ng), pSG5-SRC1e (200ng) and pCDNA-CRKL (50-200ng). After incubation for 16 hours, cells were washed and treated with 10nM hormone mibolerone (MB) for 24hours. Cells were washed twice in phosphate buffered saline (PBS) and lysed in reporter lysis buffer (Promega). Extracts were analysed for firefly luciferase activity using the LucLite™ kit (Packard) and values corrected for β-galactosidase activity measured by the GalactoLight Chemiluminescence assay (Tropix). The results shown are the averages from three independent experiments performed in duplicates ± standard error of the means.

Immunofluorescence

LNCaP, PC3 and PC3wtAR cells were grown on sterile glass coverslips in 24-well plates to 50% confluency. The relevant compounds were added to the cells and incubated for 4 hours (10nM of MB, 1μM of Cas). For transient transfection of pSVAR and pCDNA-CRKL, 200ng of the DNA were transfected in each well using FuGENE6 (Roche) according to the manufacturer's instructions. Cells were then grown for an additional 24 hours prior to treatment with the compounds. The coverslips were then washed in PBS three times, fixed in 4% paraformaldehyde for 20 minutes and solubilised in 0.2% TritonX100 for 20 minutes. The coverslips were washed three times followed by treating with 10% foetal calf serum for 45 minutes. Rabbit anti-AR (1:200) (Santa Cruz); Mouse anti-AR (1:200) (DAKO) and Mouse anti-CRKL (1:50) (Cell Signalling Technology) were added to the cells in 10% FCS for one hour. Cells were washed three times in PBS and 10% FCS added for 15 minutes before incubation with Alexa-488 conjugated goat anti-mouse (1:600) or Alexa-594 conjugated chicken anti-rabbit secondary antibody(1:600) (Molecular Probes) for one hour. After five washes in PBS, coverslips were mounted on glass slides with Vectashield containing 4'6'-diamidino-2-phenylindole (DAPI) (Vector labs). Slides were visualised on a Leica DM4000 at 100x magnification. An average of 10 images per treatment was captured.

Immunoprecipitation

Immunoprecipitation (IP) was performed as previously described[11] with some modifications. Cells were grown to 80% confluency**.** 10nM MB was added for 2 hours before harvesting. Cells were washed twice in ice-cold PBS before incubation for 20 minutes on ice in IP buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% Nonidet P-40, 1mM dithiothreitol and complete protease inhibitor cocktail). The lysates were centrifuged at 14000 rpm for 20 minutes at 4°C. The supernatant was then pre-cleared with protein-A/G-Ultralink Resin (Thermo Scientific) for 30 minutes at 4°C prior to incubation with rabbit primary antibody against AR or CRKL (Santa Cruz) overnight at 4°C. The immune complex was then precipitated with protein-A/G-Ultralink resin for 1 hour at 4°C, washed three times in PBS and resuspended in laemmli SDS loading buffer for separation by SDS-PAGE.

Western blotting

All cell extracts were prepared at a concentration of 30μg per well in SDS-PAGE loading buffer and loaded on to Novex 4-20% Tris-Glycine Gels (Invitrogen). Under denaturing conditions, proteins were separated by gel electrophoresis and transferred onto nitrocellulose membrane using a semi-dry blotting apparatus (Trans-Blot SD Semi-Dry. Biorad). The membranes were blocked in TBS containing 5% non-fat milk for 1 hour before incubating with primary antibodies for 1 hour at room temperature. Antibodies used were mouse anti-CRKL (Cell Signaling), rabbit anti CRKL (Santa Cruz), rabbit anti-AR(N20) (Santa Cruz), mouse anti-AR (DAKO), rabbit anti phospho CRKL (Tyr 207) (Cell Signaling), rabbit anti Phospho ERK (Cell Signaling) and Rabbit β-actin (Sigma). After subsequent membrane washing, detection was carried out using the horseradish peroxidase conjugated anti-mouse or anti-rabbit or mouse IgG secondary antibody (1:5000) (Jackson Immuno Research) and incubated for 1 hour at room temperature. Following further washes, proteins were visualised using the enhanced chemiluminscence detection system (Amersham).

KEY RESEARCH ACCOMPLISHMENTS

1. We have completed all the questions for Task 1.

2. We have partially completed the questions for Task 2.

3. We have identified a new molecular target for prostate cancer: CRKL/beta1 integrin. This work has been accepted by a peer-review journal *PNAS* (in press, 2008) (see appendix)

4. Another novel complex has been identified—CRKL/AR (androgen receptor).

REPORTABLE OUTCOMES

1. A paper has been accepted in a peer-review journal *PNAS* (in press, 2008) (see appendix).
2. Second manuscript in preparation, 2008.

CONCLUSIONS

We have identified a couple of new receptor-ligand complexes in prostate cancer. The association between β_1 integrin/CRKL and AR/CRKL are novel discoveries and this is the first report of such findings. The work generated in characterizing the interaction between β_1 integrin/CRKL has culminated into a paper that will be published in a peer-review journal *PNAS* (in press, 2008). Moreover, we have partially characterized the AR/CRKL interaction and we are confident that this will be completed in the coming year. We are excited with our novel findings and we hope to have another publication. Our work will have important impact on the prostate community as these findings are novel and potential new therapeutic targets to fight against prostate cancer.

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APPENDICES

1. Mintz et al. An unrecognized extracellular function for an intracellular adapter protein released from the cytoplasm into the tumor microenvironment. *PNAS* (in press, 2008).